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New method for the selective capture of antibodies under physiolgical conditions

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Abstract

Hydrophobic charge induction chromatography is a recently developed method for protein separation based on the use of dual-mode ligands. They are designed in such a way so as to combine a molecular interaction supported by a mild hydrophobic association effect in the absence of salts. When environmental pH is changed, the ligand becomes ionically charged resulting into the desorption of the protein. This method is applied to the separation of antibodies from ascite fluids and culture supernatants from hybridomas cultured in the presence of fetal bovine serum or in protein free environment. Typically adsorption from cell culture supernatants is accomplished without any pH or ionic strength adjustment; the column is then washed with a typical buffer to eliminate protein impurities. Antibodies are then desorbed using acetate buffer, pH 4. Antibody binding capacity is in the range of 30 mg per ml of resin at 10% breakthrough. Antibody purity varies according to the initial feed stock and can reach values higher than 90% in a single pass. One example of antibody purification process involving hydrophobic charge induction chromatography as a capture step followed by a polishing phase with DEAE Ceramic HyperD is described. Longevity and ligand leakage are compatible with large-scale applications.

Introduction

The growing role of monoclonal antibodies in biopharmaceutical research and development is widely acknowledged. In parallel, antibody-based therapeutics and *in vivo* diagnostics are gaining wider approval from regulatory agencies around the world. In evaluating the significance of this trend, it is important to note that antibody-based therapeutics are typically administered at relatively high dosage levels. Equally significant, new applications for these biopharmaceuticals have been emerging rapidly. Taken together, these factors suggest that requirements for production of antibody-based therapeutics may grow exponentially in the coming years.

The high dosage levels of therapeutic antibodies and the parenteral use of diagnostic antibodies need that products meet stringent requirements for purity, safety and potency. If these biopharmaceuticals are to be produced economically at required scale, effective and efficient purification strategies must be employed.

During the last two decades, a wide variety of strategies for purification of antibodies have been developed. At present, the most widely used technique for capture and initial purification is affinity chromatography on Protein A sorbents. The high specificity of Protein A for the Fc antibody domain provides excellent chromatographic selectivity and facilitates isolation of high purity products. However, as chromatographers have been called upon to design schemes for process-scale purification of antibodies, various practical complications associated with Protein-A chromatography have come under increasing scrutiny. For example, the cost of Protein A sorbents remains high despite the efforts of vendors to pass along to users the economies of scale arising from increased use of these sorbents. Equally important, Protein A sorbents can not be subjected to clean-in-place procedures using sodium hydroxide solutions preferred in process-

scale applications. Moreover, Protein A is subject to degradation by proteases present in the feedstocks. Compared to other chromatographic media, the number of use-cycles that can be achieved using Protein A sorbents is relatively limited. Leaching of Protein A (or fragments) must be addressed in the overall scheme. Finally the low pH elution of antibody can lead to aggregate formation or loss of activity. These issues have prompted process developers to investigate alternatives to chromatography on Protein A (Ngo, 1994; Scholz et al., 1998a, b). In order to avoid complications related to Protein A sorbents a number of alternative separation techniques are currently available. The literature is actually replete with references to chromatographic modes useful in antibody purification. Ion exchange chromatography (Rapoport et al., 1995), hydrophobic interaction chromatography (Manzke et al., 1997), thiophilic chromatography (Sulk et al., 1992), molecular sieving (Ostlund et al., 1987), various bio-affinity techniques (Pilcher et al., 1991; Thommes et al., 1996), dye ligand chromatography (Bruck et al., 1982) and chromatography on ligands identified by combinatorial chemistry (Li et al., 1998; Palombo et al., 1998) have all been described (for review see also Boschetti and Jungbauer, 2000).

Traditional techniques such as ion exchange chromatography, hydrophobic interaction chromatography, or thiophilic chromatography can, of course, be conducted at a fraction of the cost of affinity chromatography on Protein A. On its face, such a comparison is of little significance since these techniques lack the selectivity of Protein A. Reference to traditional techniques is relevant, however, if one wishes to evaluate a novel alternative to Protein A. Ideally, an alternative sorbent would provide selectivity approaching that of Protein A combined with the process economics usually associated with traditional techniques. Results presented below demonstrate that a recently introduced separation method named Hydrophobic Charge Induction Chromatography goes a long way towards achieving this ideal. Indeed, the operating characteristics of these related sorbent allow considerable process-simplification compared with traditional chromatographic modes. An overview of the mechanism that supports these operating characteristics is presented in the Results and discussion section.

The need to provide high purity antibodies at moderate cost has led researchers also to explore two production approaches. The first has sought to reduce purification costs by producing monoclonal antibodies using protein-free cell culture media. A second has sought to reduce feedstock costs by producing monoclonal antibodies in the milk and eggs of transgenic animals, and in transgenic plants. Isolation of antibodies from such crude feedstocks would be significantly facilitated by the availability of a robust, selective, cost-effective chromatographic sorbent. At the same time, such a sorbent would also provide important economic benefits to those developing processes based on use of cell culture in defined growth media. This study examines isolation and purification of antibodies by hydrophobic charge induction chromatography from very crude feedstocks such as ascite fluids and cell culture supernatants containing 5% fetal bovine serum and from protein-free cell culture supernatants.

Material and methods

Chemicals and biologicals

Cell culture supernatants containing antibodies were from Bioatlantic Nantes, France and ascite liquid (H12H11) was from CER Marloie, Belgium.

Chromatography sorbents for preparative applications such as MEP HyperCel (sorbent for hydrophobic charge induction chromatography) and DEAE Ceramic HyperD were from Life Technologies (Grand Island, NY, USA). MEP HyperCel is a cellulose beaded matrix on which 4-mercaptoethyl-pyridine is chemically attached. TSK-GSW-3000 column was bought from Tosoh (Japan). Electrophoresis plates composed of 12% polyacrylamide were from BioRad, Ivry sur Seine, France.

Standard proteins, fine chemicals and buffers were from Sigma Chemicals, St Louis, MO, USA.

Sorbent characterization

Binding capacity of MEP HyperCel for human polyclonal IgG was measured by frontal analysis in a column of 6.6 mm diameter and 50 mm long. Titration solution was a 0.05–5 mg/ml hIgG in phosphate-citrate buffers of different pH from 5 to 10 with a conductivity close to 5 mS/cm, or in a phosphate buffer, pH 7 containing different concentrations of sodium chloride up to 1 M. Linear flow rate was of 70 cm/h; calculations of dynamic binding capacity were made at 10% breakthrough.

Feed stocks

Feed stocks used for the capture and separation of antibodies were an ascite fluid, a cell culture supernatant containing 5% fetal bovine serum and a protein-free cell culture supernatant.

The mouse ascite fluid contained IgG2a antibody at a concentration of 3.2 mg/ml. It was used after filtration and dilution twice with the loading buffer (50 mM Tris-HCl, pH 8).

The cell culture supernatant containing 5% fetal bovine serum was from a hybridoma culture. The expressed antibody was an IgG1; its concentration was 100 μg/ml. This feed stock was filtered to remove suspended particulate matter and was loaded on the column without further treatment.

The serum-free cell culture supernatant was also from a hybridoma cell culture. It contained 114 μg/ml expressed IgG1 antibodies and was loaded directly in the column with no further modification.

Chromatographic separations

Capture and separation of antibodies was performed using MEP-HyperCel packed on columns; further flow-through purification of the antibody was also performed using a column of DEAE Ceramic HyperD.

MEP HyperCel was packed into a glass column of 6.6 or 11 mm diameter and 100 mm in length. It was washed extensively with a buffer sequence used for the chromatographic separation and finally equilibrated in a phosphate buffered saline (25 mM phosphate containing 150 mM sodium chloride, pH 7.4). The column was then loaded with three different crude samples.

(a) Ascite fluid: the loaded volume was 2.8 ml diluted with an equal volume of loading buffer; this corresponded to 9 mg of antibody.
(b) Protein-free cell culture supernatant: the loading volume was 150 ml; this corresponded to 17.1 mg of antibody.
(c) Cell culture supernatant containing 5% fetal bovine serum: the loading volume was also 300 ml corresponding to 30 mg of antibody.

After loading the columns were washed with 50 mM Tris-HCl buffer, pH 8 to wash out protein impurities.

In the case of ascite fluid as well as of fetal bovine serum-containing cell culture supernatant, albumin was also partially captured by the MEP-HyperCel; it was desorbed by a wash with a 25 mM sodium caprylate solution in the starting buffer followed by a distilled water wash. In all cases bound antibodies were desorbed using 50 mM acetate buffer, pH 4.0. When elution was achieved, the column was cleaned with either 0.1 or 1 M sodium hydroxide and then re-equilibrated with the starting buffer.

Analytical determinations of chromatographic fractions

Collected fractions were analyzed by SDS-polyacrylamide gel electrophoresis under both reducing and non-reducing conditions. Analysis by GPC-HPLC were performed on a 4 μm TSK-GSW3000 column of 30 cm in length and 4.6 mm diameter. Flow rate was 0.2 ml/min.

Sorbent stability over repeated use and alkaline washings

A column of 6.6 mm diameter and 100 mm in length was packed with MEP HyperCel and equilibrated with a phosphate buffered saline. A sample of 2 ml of protein free cell culture medium containing 5 mg/ml IgG was then loaded into the column. A washing was performed using 2 column volumes of 50 mM Tris-HCl buffer, pH 8 followed by the elution of antibodies using a 50 mM acetate buffer, pH 4. After the collection of the antibody fraction for further analysis, the column was cleaned with two column volumes of 1 M sodium hydroxide. This cycle was repeated for 204 times. At the issue of the experiment the IgG binding capacity was measured before and after a regeneration of the column with two volumes of 2 M urea.

Ligand release quantification from the solid phase

Two series of ten separation cycles of antibodies were performed as described above. In one series the cleaning was performed using 1 M sodium hydroxide; in the other, 6 M guanidine-HCl, pH 6 was used instead as cleaning agent. The desorbed antibody fractions at pH 4 were collected and the concentration of antibody determined classically by spectrophotometry. Each fraction was then analyzed for its content of 4-MEP free ligand. For this purpose a 2 μm Polysphere HPLC analytical column (4.6 ID $\times$ 50 mm in length) was used.

Each single antibody sample (2 ml) was spiked with different amounts of pure 4-MEP, mixed thoroughly and the proteins were precipitated by adding 0.4 ml trichloroacetic acid. 50 μl of the supernatant was then used as column load. The starting buffer was a 25 mM Tris-HCl, pH 6.2 and the elution was

performed by a gradient with acetonitrile up to 50% concentration. Absorbance wavelength was 254 nm.

A dose-response graphic was made and the content of free 4-MEP in the antibody fraction determined. Sensitivity of the method was estimated of 0.5 ppm.

Results and discussion

Efficient, economic capture of monoclonal antibodies directly from crude feedstocks is a critical element in modern schemes for process-scale purification of these biomolecules. In this connection, process development scientists recognize that the ionic strength of typical feedstocks is too high to permit capture by most ion exchange media. Recently, a special cation exchange sorbent was developed that provides for efficient binding at physiological ionic strength (Necina et al.,1998). However, even with this sorbent, pH of the feedstock must be adjusted prior to loading. Adjustment of feedstock composition is also required if antibody capture is to be conducted by either hydrophobic interaction chromatography or thiophilic chromatography: a suitable concentration of lyotropic salt must actually be added. Until recently, only bioaffinity chromatography on Protein A or Protein G sorbents allowed for direct capture of antibodies from unadjusted feedstock at near-neutral pH and physiological ionic strength. A comprehensive review (Boschetti and Jungbauer, 2000) and related publications (Nopper et al., 1989; Kent, 1994; Necina et al., 1997) describe adjustments to feedstock composition that must be made when various chromatographic modes are employed. Such adjustments, whether by dilution, adjustment of pH or addition of salt, lead to significant increases in process costs. Moreover, adjustment of feedstock pH can sometimes lead to precipitation of antibody. Hydrophobic charge induction chromatography provides for antibody capture without need for adjustment of pH or ionic strength of typical feedstocks. Like bioaffinity chromatography on Protein A or Protein G, capture is accomplished under near-physiological conditions. With hydrophobic charge induction chromatography, however, sorbent and process costs are substantially less than those of bioaffinity chromatography.

In order to best appreciate the benefits of hydrophobic charge induction chromatography, one must examine the mechanism that underlies the technique. As described by Burton and Harding (1998), hydrophobic charge induction chromatography is based on

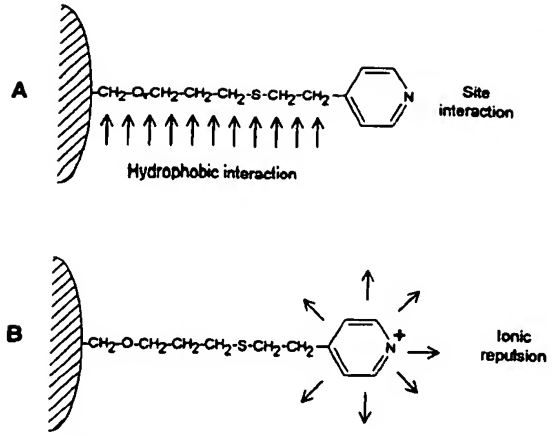

Figure 1. Schematic tentative representation of protein interaction mechanism with MEP HyperCel. (A) In physiological pH pyridine ring is not charged thus interaction occurs by a mild hydrophobic association with the whole ligand. (B) When environmental pH confers to pyridine ring a positive charge the protein (same charge) is desorbed by repulsion.

the pH-dependent behavior of dual-mode, ionizable ligands (e.g., pyridine derivatives). Over a pH range 6–9, the ligand is uncharged. Under these conditions, the ligand and the spacer arm behave much like a hydrophobic site and bind the protein by hydrophobic association. The sorbent is designed (e.g., ligand and spacer-arm structure, ligand density) so that binding occurs without need for addition of lyotropic salt. When pH of the mobile phase is modified, the ligand takes a net ionic charge. Under these conditions, the antibody also carries a similar net positive charge, therefore desorption occurs on the basis of electrostatic repulsion between the solid phase sorbent and the protein (see Figure 1). In contrast to hydrophobic interaction chromatography or thiophilic chromatography, hydrophobic charge induction chromatography is controlled on the basis of pH rather than salt concentration. Beyond its role as hydrophobic ligand, the pyridine-derivative employed here (4-mercaptoethyl pyridine or 4-MEP), has enhanced specificity for immunoglobulins compared to a simple phenyl group. The selectivity of various nitrogen-heterocycles was demonstrated in studies by Oscarson and Porath, 1990 and Schwarz, 1996. Selectivity is further enhanced by the presence of a sulfur atom proximal to the pyridine ring. Analogous ligands have recently been described as improved compounds for the adsorption of antibodies in a salt independent manner (Scholz et al.,

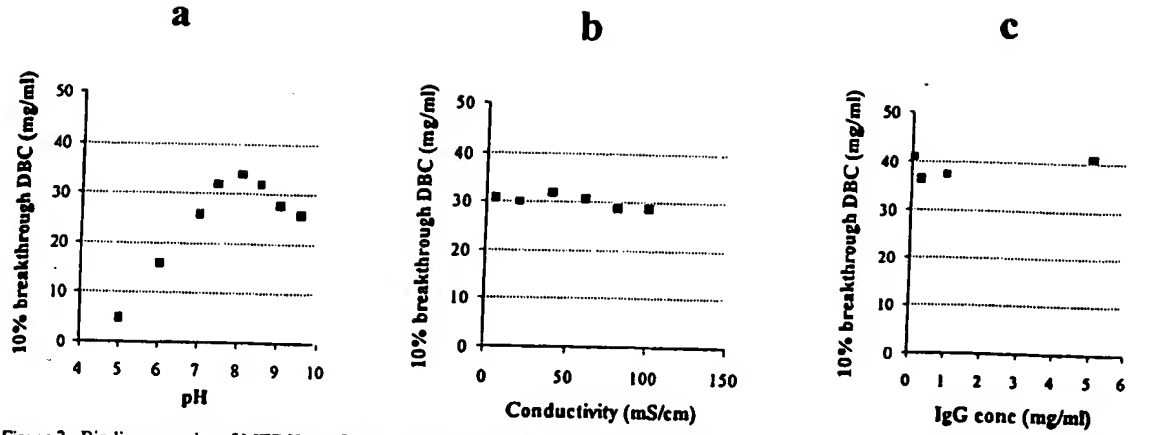

Figure 2. Binding capacity of MEP HyperCel for human polyclonal IgG in various conditions of pH, ionic strength and antibody concentration. All determinations were performed in column (6.6 mm ID × 400 mm) by frontal analysis and calculations make at 10% breakthrough. (A) IgG concentration: 5 mg/ml; buffers: phosphate-citrate with conductivity of 5 mS/cm and pH from 5 to 10. (b) IgG concentration: 5 mg/ml; buffer: 25 mM phosphate, pH 7 containing various amount of sodium chloride up to 1 M. (c) IgG concentration from 0.05 to 5 mg/ml; buffer: 25 mM phosphate, pH 7 containing 0.15 M sodium chloride.

1998a, b). These ligands combine a traditional thiophilic structure (i.e., a divinyl sulfone group) with heterocyclic structures such as 3-(2-mercaptoethyl)-quinazoline-2,4-(1H,3H)dione, 2-mercaptopyrimidine and 2-mercaptonicotinic acid (Finger et al., 1996).

In developing the sorbent described here, 4-MEP was chosen on the basis of both selectivity and pKa. The pKa of the ligand is 4.8. At pH <4.8, the ligand takes on a predominant positive charge. As a result, the pH of the mobile phase needs only be reduced to 4.0–4.8 for desorption to occur. During affinity chromatography on Protein A, desorption is typically conducted at pH 3; under these conditions, aggregation or loss of activity may occur. In comparison, the method described here provides for significantly milder desorption conditions than those employed with Protein A.

Typical feedstocks have a pH near neutrality and ionic strength in the range of 13–16 mS/cm. As noted above, the ionic strength is too low to permit direct loading of unadjusted feedstock on to a hydrophobic interaction sorbents (Gooding et al., 1986) or thiophilic sorbent (Sulk et al., 1992). At the same time the ionic strength is too high for direct loading onto an ion exchanger. With hydrophobic charge induction chromatography however, the behavior is somewhat different: it does not necessitate adjustments in pH and ionic strength.

The influence of pH and of ionic strength on dynamic binding capacity (at 10% breakthrough) using MEP-HyperCel as sorbent is illustrated in Figure 2a,b. In the domain of pH 6.5 to 9.0, binding capacity ranges from approximately 20–33 mg of human polyclonal IgG per ml of sorbent. Below pH 6.5, binding capacity decreases rapidly. The pH dependence observed is just as expected given the pKa of the ligand and the proposed adsorption-desorption mechanism. As shown binding capacity is independent of ionic strength over a broad range (5–100 mS/cm).

Data in Figure 2c demonstrate that under physiological conditions (pH 7.2 and conductivity of 15 mS/cm), dynamic binding capacity is independent on antibody concentration over a one hundred fold range (50 μg/ml to 5 mg/ml).

In this study the effectiveness of the sorbent to capture selectively antibodies is demonstrated starting from different feed stocks. IgG was isolated from ascite fluids, cell culture supernatant containing 5% fetal bovine serum, and protein-free cell culture supernatant. Figure 3 illustrates the isolation of IgG_{2a} from ascites fluid. Before application the viscous sample was diluted with an equal volume of loading buffer (50 mM Tris-HCl buffer, pH 8) and filtered. The IgG_{2a} was fully retained – none was detected in the load flow-through fraction, or during further wash with loading buffer. A quantity of albumin was also retained. From the chromatogram and accompanying SDS-PAGE results, it is clear that most of the impurity protein is unretained during the load and initial wash. Further washes with 25 mM sodium caprylate

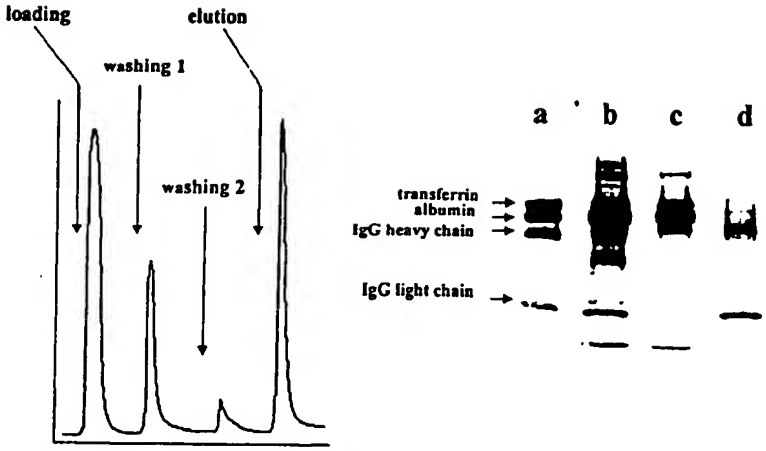

Figure 3. IgGa separation from a crude ascite fluid on MEP-HyperCel column. Sample: 2.5 ml of ascite fluid diluted with an equal volume of 50 mM Tris-HCl buffer, pH 8; Column: 6.6 mm ID × 100 mm; equilibration buffer: 50 mM Tris-HCl, pH 8; Washing solutions: 25 mM sodium caprylate in 50 mM Tris-HCl buffer, pH 8, followed by distilled water; Eluting solution: 50 mM acetate buffer, pH 4.0; Cleaning solution: 1 M sodium hydroxide; Linear flow rate: 70 cm/h. Collected fractions were analyzed by SDS-PAGE in reduced conditions and by GPC-HPLC. For more details see Material and method section. (a) Molecular markers; (b) crude asite fluid; (c) flowthrough fraction; (d) elution of antibodies.

in loading buffer (wash-1) and pure water (wash-2) were conducted. During washes 1 and 2 a significant quantity of protein impurities were desorbed (data not shown). The mild detergent-like properties of sodium caprylate promote desorption of hydrophobic species such as the weakly-bound albumin. Following the wash sequence, IgG2a was eluted under the influence of 50 mM sodium acetate buffer, pH 4. Based on SDS-PAGE analysis, purity of the immunoglobulin fraction was estimated to be about 83%.

These findings were confirmed by high performance gel filtration chromatography, shown in Figure 4. Recovery of immunoglobulin was about 79%; the purification factor was 5. Studies were continued with isolation of IgG from cell culture supernatant containing 5% FBS and protein-free cell culture supernatants. In both cases, IgG concentration was low (100 and 114 mg IgG_1/ml). Cell culture supernatants were filtered before loading, but was employed without dilution or preliminary adjustment of pH or ionic strength.

In the sample containing 5% FBS, albumin was the most abundant protein present. During loading phase, all IgG were captured along with some albumin. Accordingly, the chromatographic sequence included washes with 25 mM sodium caprylate and with pure water, as described above for chromatography of ascites fluid to increase the purity of eluted antibodies. In contrast, isolation of IgG from protein-free cell

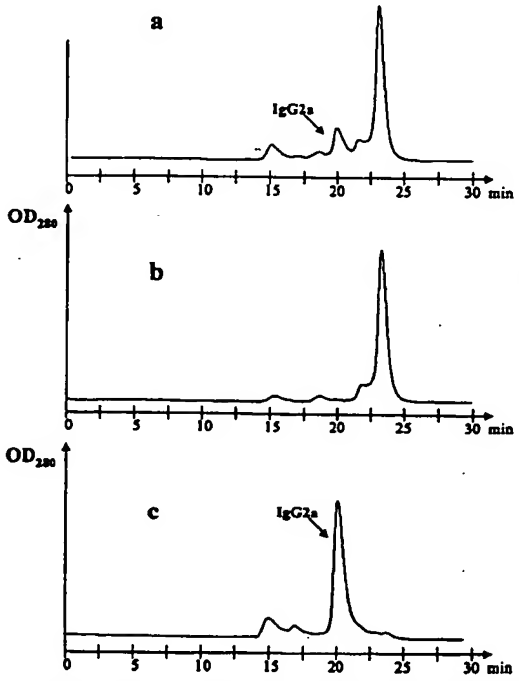

Figure 4. GPC-HPLC analysis of fractions collected from MEP-HyperCel chromatography of ascite fluid (see Figure 3). Samples volume: 20 μl; Column: TSK-GSW 3000 prepacked (4.6 mm ID × 300 mm), 4 μm particle diameter; Running buffer: 25 mM phosphate, 0.15 M sodium chloride, pH 7.4; Flow rate: 2 ml/min. (a) Crude ascite fluid; (b) flowthrough; (c) elution fraction.

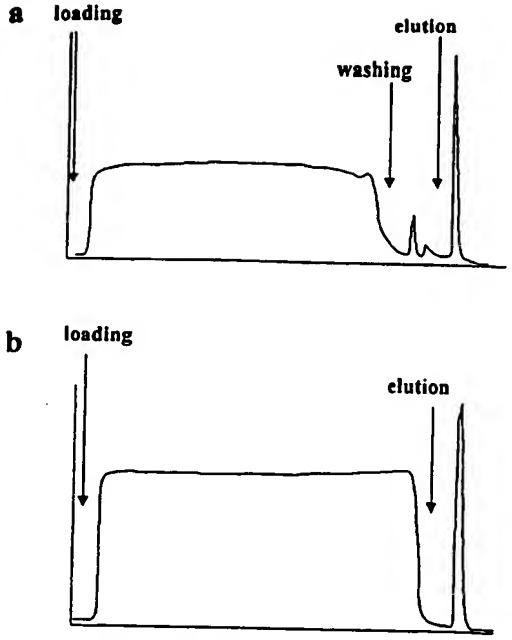

Figure 5. Purification of IgG1 from cell culture supernatants on MEP-HyperCel columns. Columns: 11 mm ID × 100 mm; Samples: (a) the cell culture medium contained 5% fetal bovine serum (b) the cell culture supernatant was protein free. Equilibration buffer: 25 mM phosphate, 0.15 M sodium chloride, pH 7.4; Washing solutions (only for serum containing cell culture supernatant): 25 mM sodium caprylate in 50 mM Tris-HCl buffer, pH 8, followed by distilled water; Eluting solution: 50 mM acetate buffer, pH 4.0; Cleaning solution: 1 M sodium hydroxide; Linear flow rate: 70 cm/h. Collected fractions were analyzed by SDS-PAGE in reduced conditions and by GPC-HPLC. For more details see Material and method section.

culture supernatant was conducted without these two additional wash steps. Chromatographic separations are shown in Figure 5. Chromatographic fractions were analyzed using high performance gel filtration chromatography (see Figure 6) and SDS-PAGE (data not shown).

Examination of Figures 5 and 6 reveal that a large mass of impurity protein was unretained, appearing in the load/wash flow-through fraction. Antibody capture was efficient and complete: no IgG appeared in the load/wash flow-through fraction. Again, washes with 25 mM sodium caprylate facilitated removal of a large amount of bound impurities (mainly albumin) during chromatography of fetal bovine serum-containing sample. As shown in Figure 5, IgG was eluted under the influence of 50 mM sodium acetate, pH 4. IgG isolated from cell culture supernatant containing 5% fetal bovine serum was approximately 76% pure (albumin was the principal impurity) while that isolated from protein-free cell culture supernatants was approximately 99% pure (values based on SDS-PAGE analysis). Purification factors were approximately 40 and 19, respectively. In both cases the concentration factor was substantial; additionally the concentration of IgG in the eluted fraction was approximately 20-fold greater than that in the feedstock. Table 1 summarizes most relevant data from antibody separation.

Most generally schemes for antibody purification involve at least two (Sene et al., 1990), but frequently three or more (Duffy et al., 1989) complimentary/orthogonal chromatographic steps. This is a well-established practice in processes intended for production of therapeutic products. In such applications, robust, multi-step chromatographic schemes are employed even when initial purification by affinity chromatography provides good purity products. Under optimal conditions affinity chromatography may yield product of 95–99% purity. In some applications, such as isolation of IgG from protein-free cell culture supernatant, hydrophobic charge induction chromatography, too, can provide product of 95–99% purity in a single step.

In order to provide a measure of the practical utility of the antibody separation method described here, a typical multi-step scheme findings are presented below for isolation of monoclonal IgG from cell culture supernatant containing 5% fetal bovine serum. Two columns were used: the first was MEP HyperCel followed by anion exchange chromatography on DEAE Ceramic HyperD. The justification of this column is because anion exchange chromatography is frequently included in IgG purification sequences to bind protein impurities, residual DNA and endotoxin.

As shown in Figure 6 the IgG fraction was easily separated from the crude feed stock. Analysis by SDS-PAGE indicated that the product was approximately 70% pure. This fraction was adjusted to pH 8.8, sodium chloride was added to bring the conductivity of the solution to 8 mS/cm (~100 mM) and then applied to the anion exchange column (previously equilibrated in a buffer of the same pH and ionic strength). As shown in Figure 7, the IgG fraction was unretained. Protein impurities (principally albumin) along with a small quantity of IgG were bound and subsequently eluted under the influence of 50 mM Tris-HCl buffer, pH 8.8, containing 1 M sodium chloride. Analysis of the IgG target fraction by SDS-PAGE (see Figure 7b) indicated that the product was at least 97% pure –

Table 1. Summary of main separation results of monoclonal antibodies from different feedstocks

Feedstock	Total proteins (mg)	Mab content (mg)	Man initial purity (%)	Mab final purity (%)	Mab recovered (mg)	Overall Mab yield (%)	Purification factor
Ascite fluid	55	9	16	83	7	79	5
Serum free supernatant	330	17.1	5	99	17	99	19
Supernatant with 5% fetal serum	1740	30	1.7	69	23	76	40

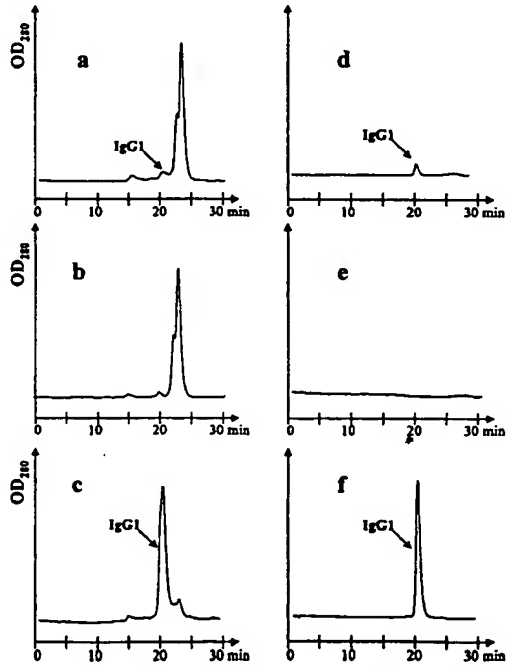

Figure 6. GPC-HPLC analysis of fractions collected from MEP-HyperCel chromatography of cell culture supernatants (see Figure 5). Samples volume: 20 μl; Column: 3000 prepacked (4.6 mm ID x 300 mm); 4μm particle diameter; Running buffer: 25 mM phosphate, 0.15 M sodium chloride, pH 7.4; Flow rate: 2 ml/min. (a) whole cell culture supernatant containing 5% fetal bovine serum; (b) IgG1-depleted cell culture supernatant containing 5% fetal bovine serum; (c) elution fraction from cell culture supernatant containing 5% fetal bovine serum; (d) whole protein free cell culture supernatant; (e) IgG1-depleted protein free cell culture supernatant; (f) elution fraction from protein free cell culture supernatant.

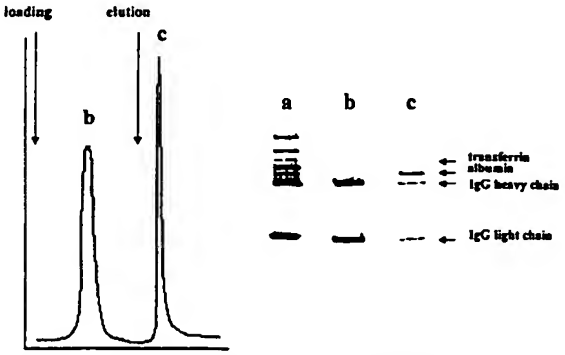

Figure 7. Isolation if IgG1 from a prepurified fraction on a DEAE Ceramic HyperD column. Columns: 11 mm ID x 100 mm equilibrated with 25 mM Tris-HCl buffer, pH 8.8 containing sodium chloride to give a conductivity of 10 mS/cm. Sample: IgG fraction from MEP HyperCel. Initial feed stock was a cell culture supernatant containing 5% fetal bovine serum. The sample was equilibrated with the column buffer. Eluting solution: 25 mM Tris-HCl buffer, pH 8.8 containing 0.5 M sodium chloride; cleaning solution: 0.1 M sodium hydroxide; Linear flow rate: 70 cm/h. Collected fractions (b) and (c) were analyzed by SDS-PAGE in reduced conditions. For more details see Material and method section.

a substantial enhancement in purity. Advantages related to the use of anion exchange chromatography are not only related to the purity of the antibodies. Since the anion exchange column is employed solely to bind impurity components, the column volume required is modest. The process-cost of including this simple anion exchange procedure in a scheme would be equally modest. As a practical matter, anion exchange chromatography can not only bind residual DNA and endotoxin, but can also be employed to remove residual albumin (or other acidic impurities) from the IgG fraction harvested using MEP HyperCel.

Economic process-scale use of a chromatographic sorbent is significantly facilitated if the sorbent is compatible with clean-in-place procedures preferred in cGMP operation. Accordingly, MEP HyperCel was evaluated over a series of 204 chromatographic cycles. Each cycle (see Materials and methods section) included application of a IgG-containing sample at pH 8, elution at pH 4, wash with 1 M sodium hydroxide

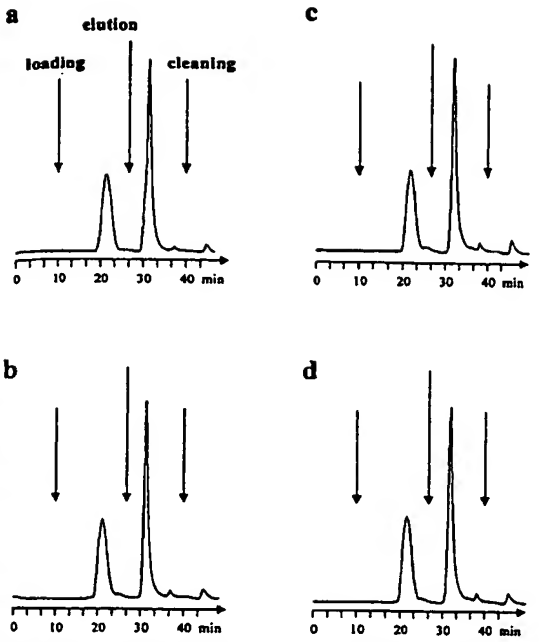

Figure 8. Chromatographic profiles from stability study on repeated separation cycles. Column: 6.6 mm ID × 100 mm; sample: protein free cell culture supernatant containing 5 mg/ml human IgG; loading buffer: 50 mM Tris-HCl, pH 8.0; elution buffer: 50 mM acetate, pH 4.0; cleaning solution: 1 M sodium hydroxide. (a) cycle 1; (b) cycle 100; (c) cycle 150; (d) cycle 204.

and re-equilibration in preparation for the next cycle. Chromatograms for cycles 1, 100, 150 and 204 are shown in Figure 8. There were no significant changes in the chromatographic profiles over the series. Recovery of IgG averaged at least 90%, with no significant decline over the series. At the conclusion of the sequence, dynamic binding capacity was re-determined (frontal analysis at 10% breakthrough) and compared with the value determined initially. Binding capacity had declined to 26 mg IgG/ml from the initial value of 34 mg IgG/ml. Experiments were conducted to ascertain the reason for this behavior. Since such a decline could be attributed to loss of ligand over the series, ligand density was re-determined and compared to the original value. Within limits of measurement, the two values were identical: 109 μmol/ml at the end of the sequence compared with 107 μmol/ml determined initially. Next, the resin was washed with 2 M urea to determine if the reduction in binding capacity might be attributed to incremental fouling over the series. Binding capacity was fully restored following the urea wash. It was concluded that some hydrophobic foulants from the crude feed stock were not efficiently removed by the sodium hydroxide wash. Thus, occasional/periodic use of a urea wash may be advisable in various applications.

Further experiments were conducted to assess the chemical stability of the linkage between ligand and solid support. Two series of ten chromatographic cycles were conducted. In the first series each cycle included a wash with 1 M sodium hydroxide, whereas the second series included a wash with a aqueous solution of 6 M guanidine hydrochloride, pH 6. IgG fractions were collected and analyzed for the presence of 4-MEP using reversed-phase HPLC. The detection limit of the procedure was 0.5 ppm 4-MEP in IgG, wt./wt. (see Materials and methods section). Following the first separation in the sequence, the concentration of 4-MEP was approximately 1 ppm. In the subsequent nine separations, 4-MEP was below the limit of detection for both series. The concentration of 4-MEP in the first cycle may reflect insufficient washing of the sorbent prior to chromatography.

The above determinations of 4-MEP in collected IgG fractions should be considered together with experiments demonstrating that ligand density was unchanged after 204 sodium hydroxide wash cycles. Taken together, the data demonstrate that the linkage between the ligand and the support is highly stable in the presence of strong cleaning agents. However important the above performance characteristics may be, they would be of limited value in the absence of high selectivity. As demonstrated above, IgG can be isolated from crude feedstocks (e.g., ascites fluid or cell culture supernatants containing 5% fetal bovine serum) at purity of ~70–80% in a single chromatographic step. If IgG is isolated from protein-free cell culture supernatant, corresponding purity of 95–99% can be obtained. Product of such purity cannot typically be obtained in a single step when capture is performed using traditional chromatographic modes such as ion exchange or hydrophobic interaction chromatography.

Work-in-progress indicates that hydrophobic charge induction chromatography provides effective purification of IgG from other crude feedstocks such as egg yolk and bovine colostrum. Future studies will be focused on the isolation of monoclonal IgG from transgenic milk and from extracts of transgenic plant material.

Conclusion

Hydrophobic charge induction chromatography, conducted here using a sorbent bearing a 4-MEP ligand, provides effective capture of IgG from both crude feedstocks and protein-free cell culture supernatant. Feedstock may be applied to the column without adjustment of pH or ionic strength, without addition of lyotropic salt, and without preliminary concentration. The sorbent provides high dynamic binding capacity (~30 mg IgG/ml at 10% breakthrough). The 4-MEP ligand shows a high degree of selectivity for IgG – considerably higher than that typically provided by traditional ion exchange or hydrophobic interaction sorbents.

Depending on feedstock composition, IgG purity ranging from approximately 70–99% may be achieved in a single chromatographic step. Elution of IgG is achieved under relatively mild conditions (pH 4.0–4.5) compared to conditions employed during affinity chromatography on Protein A sorbents. The likelihood of aggregate formation or inactivation is thus reduced. Finally, crude feedstocks containing high concentrations of albumin can be brought to high levels of purity (>97%) by use of a simple anion exchange procedure following hydrophobic charge induction chromatography.

The sorbent is chemically stable in the presence of 1 M sodium hydroxide – the clean-in-place solution preferred in cGMP operation. No reduction in ligand density was observed over a large series of chromatographic cycles, each including a caustic wash. Little or no ligand leakage was observed in a related sequence of multiple chromatographic cycles. It is anticipated that use of hydrophobic charge induction chromatography for antibody capture using immobilized 4-MEP will substantially reduce process-costs compared to capture using bioaffinity chromatography on Protein A sorbents. Favorable process economics will play an increasingly important role as therapeutic and diagnostic applications of monoclonal antibodies continue to expand. In this respect studies concerning isolation of antibodies from bovine sweet whey and other crude feedstocks are in progress.

References

Boschetti E and Jungbauer A (2000) Separation of antibodies by liquid chromatography. Handbook of Bioseparation, Academic Press, S. Ahuja ed., vol. 2 pp 533–632.

Bruck C, Portelle D, Glineur C and Bollen A (1982) One-step purification of mouse monoclonal antibodies from ascites fluid by DEAE Affigel Blue chromatography. J. Immunol. Meth. 53: 313–319.

Burton SC and Harding DRK (1998) Hydrophobic charge induction chromatography: salt independent protein adsorption and facile elution with aqueous buffers. J. Chromatogr. A. 814: 71–81.

Duffy S, Moellering BJ, Prior GM, Doyle KR and Prior CP (1989) Recovery of therapeutic-grade of antibodies: Protein A and ion exchange chromatography. BioPharm. 6: 35–47.

Finger UB, Brummer W, Knieps E, Thommes J and Kula MR (1996) Investigations on the specificity of thiophilic interaction for monoclonal antibodies of different subclasses. J. Chromatogr. 675: 197–204.

Gooding DL, Schmuck MN, Nowlan MP and Gooding KM (1986) Optimization of preparative hydrophobic interaction chromatography purification methods. J. Chromatogr. 359: 331–337.

Kent UM (1994) Purification of antibodies using ion exchange chromatography. Methods Mol. Biol. 34: 23–27.

Li R, Dowd V, Stewart DJ, Burton SJ and Lowe CR (1998) Design, synthesis and application of a Protein A mimetic. Nature Biotechnology 16: 190–195.

Manzke O, Tesch H, Diehl V and Bohlen H (1997) Single-step purification of biospecific monoclonal antibodies for immunotherapeutic use by hydrophobic interaction chromatography. J. Immunol. Meth. 208: 65–73.

Necina R, Amatschek K and Jungbauer A (1998) Capture of human monoclonal antibodies from cell culture supernatant by ion exchange media exhibiting high charge density. Biotechnol. Bioeng. 60: 679–698.

Ngo TT (1994) Rapid purification of immunoglobulin G using aza-arenophilic chromatography: novel mode of solid phase interaction. J. Chromatogr. 662: 351–356.

Nopper B, Kohen F and Wilchek M (1989) A thiophilic adsorbent for the one-step high-performance liquid chromatography purification of monoclonal antibodies. Anal. Biochem. 180: 66–71.

Oscarson S. and Porath J (1990) Protein chromatography with pyridine- and alkyl-thioether-based agarose adsorbents. J. Chromatogr. 499: 235–247.

Ostlund C, Borwell P and Malm B (1987) Process-scale purification from cell culture supernatants: monoclonal antibodies. Dev. Biol. Stand. 66: 367–375.

Palombo G, Verdoliva A and Fassina G (1998) Affinity purification of immunoglobulin M using a novel synthetic ligand. J. Chromatogr. 715: 137–145.

Pilcher JB, Tsang VCW, Zhou W, Black CM and Sidnam C (1991) Optimization of binding capacity and specificity of protein G on various solid phase matrices for immunoglobulins. J. Immunol. Meth 136: 279–286.

Rapoport EM, Zhigis LS, Vlasova EV, Piskaref VE, Bovin NV and Zubov VP 1995) Purification of monoclonal antibodies to Le(y) and Le(d) carbohydrate antigens by ion exchange and thiophilic adsorption chromatography. Bioseparation 5: 141–146.

Scholz GH, Vieweg S, Leistner S, Seissler J, Scherbaum WA, Huse K (1998) A simplified procedure for the isolation of immunoglobulins from human serum using a novel type of thiophilic gel at low salt concentration. J. Immunol. Meth. 219: 109–118.

Scholz GH, Wippich P, Leistner S and Huse K (1998) Salt-independent binding of antibodies from human serum to thiophilic heterocyclic ligands. J. Chromatogr. 709: 189–196.

Schwarz A (1996) Five-membered mercaptoheterocyclic ligands for thiophilic adsorption chromatography. J. Mol. Recognit. 6: 672–674.

Sene C, Santambien P and Pouradier Duteil X (1990) Chemistry Today 3: 15–21.

Sulk B, Birkenmeier G and Kopperschlager G (1992) Application of phase partitioning and thiophilic adsorption chromatography to the purification of monoclonal antibodies from cell culture fluids. J. Immunol. Meth. 149: 165–171.

Thommes J, Bader A, Halfar M, Karau A and Kula MR (1996) Isolation of monoclonal antibodies from cell containing hybridoma broth using a Protein A coated adsorbent in expanded beds. J. Chromatogr. 752: 111–122.

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